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**DIETARY RESISTANT STARCH INCREASES HYPOTHALAMIC POMC
EXPRESSION INDEPENDENT OF CAPSAICIN-SENSITIVE NEURONS IN
RATS**

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science**

In

The School of Human Ecology

By

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Bachelor of Medicine, Shanghai Second Medical University, China, 1998

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August 2008

DEDICATION

I would like to dedicate this to my major professor Dr. Martin and my family.

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During my two-year study in LSU, many people have provided me with support and encouragement. Without their contribution, this project wouldn't have been completed successfully. Therefore, I would like to send my sincere appreciation to:

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TABLE OF CONTENTS

DEDICATION.....	ii
ACKNOWLEDGEMENTS.....	iii
ABBREVIATIONS.....	v
ABSTRACT.....	vi
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: REVIEW OF LITERATURE.....	4
Resistant Starch.....	4
Glucagon-like Peptide-1 (GLP-1).....	6
Peptide YY (PYY).....	7
Neuropeptides in Hypothalamic Arcuate Nucleus.....	9
Capsaicin.....	11
CHAPTER 3: DIETARY RESISTANT STARCH INCREASES HYPOTHALAMIC POMC EXPRESSION INDEPENDENT OF CAPSAICIN-SENSITIVE NEURONS IN RATS.....	13
Introduction	13
Materials and Methods.....	14
Results.....	21
Discussion.....	24
CHAPTER 4: CONCLUSIONS	31
REFERENCES.....	32
VITA.....	39

ABBREVIATIONS

AGRP	Agouti-related peptide
ARC	Arcuate nucleus
CCK	Cholecystokinin
GLP-1	Glucagon-like peptide-1
MC4	Melanocortin receptor 4
NPY	Neuropeptide Y
POMC	Pro-opiomelanocortin
PYY	Peptide YY
RS	Resistant starch
VR1	Vanilloid receptor subtype 1

ABSTRACT

Resistant starch (RS) is fermentable dietary fiber. It has been shown that inclusion of resistant starch in the diet causes decreased body fat accumulation and altered gut hormone profile. Gut hormone has complex effect on neuropeptides' expression in the brain hypothalamic area which is regarded as key factors in regulation of energy homeostasis. In this project, thereby, it is proposed that 1) the hypothalamic neuropeptide Y (NPY), agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) mRNA expression may be altered by RS feeding; 2) afferent vagal nerves might be involved in this process.

Animal experiment was conducted to investigate the hypothesis. The rats were injected intraperitoneally with capsaicin to destroy unmyelinated small vagal afferent nerve fibers. The cholecystokinin food suppression test was performed to validate the effectiveness of the capsaicin treatment. Then, capsaicin treated rats and vehicle treated rats were subdivided into a control diet or a resistant starch diet group, and fed the corresponding diet for 65 days. At the end of study, body fat, food intake, plasma peptide YY (PYY) and glucagon-like peptide -1 (GLP-1), and hypothalamic pro-opiomelanocortin, neuropeptide Y, agouti-related peptide gene expressions were measured.

Resistant starch fed rats had decreased body fat, increased POMC expression in the hypothalamic arcuate nucleus, and elevated plasma PYY and GLP-1 in both the capsaicin and vehicle treated rats. Hypothalamic NPY, AgRP gene expressions and food intake were not changed by resistant starch or capsaicin. Therefore, destruction of the capsaicin sensitive afferent nerves did not alter the response to resistant starch in rats. The conclusion is that dietary resistant starch might reduce body fat through increasing

the hypothalamic POMC expression and vagal afferent nerves are not involved in this process.

CHAPTER 1

INTRODUCTION

The worldwide prevalence of obesity has risen dramatically during the past two decades, especially in the United States. The 1998-2002 National Health and Nutrition Examination Survey (NHANES) showed 65.1% of the U.S. adults aged at least 20 years are over the normal weight, among which 29.8% were overweight, 30.4% were obese and 4.9% were extremely obese (NHANES 2002). Obesity, as a chronic disease per se, has a profound impact on human health and lifespan. It leads to the increased morbidity, decreased life quality and shortened life span. Obesity is also a risk factor for many other diseases. Current evidence show that excess weight gain may be responsible for 65-75% of the risk for essential hypertension and cardiovascular disorders (Hall et al.2003). Moreover, obesity is considered as one of the main causes of type 2 diabetes (Mokdad et al. 2003). Obesity and its related diseases are costly; in 2003, the cost of obesity related diseases reached \$75 billion in USA and continued going up (CDC, 2004). Therefore, it is important and urgent to find effective ways to treat obesity.

Resistant starches are non-digestible fermentable dietary fibers that resist digestion in the small intestine, but are fermented in the large intestine. It has been shown that adding resistant starch to diets produces several health benefits, including lower body fat storage (Brown, 2004; Higgins, 2004). However, the current understanding of the mechanism of decreased body fat by resistant starch is incomplete. In addition to the conventional effects as dietary fiber, such as diluting the energy density of the diet and causing discomfort in the gut, resistant starch fed animals were also found having significantly higher levels of peptide YY (PYY) and glucagon-like peptide -1 (GLP-1) (Keenan, et al. 2006; Zhou et al. 2006). PYY and GLP-1 are gut satiety hormones and

candidates for anti-obesity drugs (Murphy et al. 2006; Young 2006). These two hormones reduce food intake and body weight by sending signals from the gut to the brain, and changing hypothalamic neuropeptide expression (Wren et al. 2007).

There are two different sets of neurons: neuropeptide Y/agouti-related peptide (NPY/AgRP) and pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus. These are the key factors in modulating energy homeostasis. Activation of POMC neurons increases energy expenditure, and activation of NPY/AgRP neurons increases food intake (Schwartz et al. 2004; Cone et al. 2001). Studies show PYY and GLP-1 affect the activities of hypothalamic NPY/AgRP and POMC neurons (Riediger et al. 2004; Acuna-Goycolea et al. 2005; Larsen et al. 1997; Ghamari-Langroudi et al. 2005). Peripheral injection of PYY decreases NPY mRNA (Batterham et al. 2002; Challis et al. 2003) and increases POMC mRNA in the hypothalamus (Challis et al. 2003). The modulation of NPY/AgRP and POMC neurons by PYY or GLP-1 can be directly or through vagal nerves (Abbott et al. 2005; Koda et al. 2005; Osaka et al. 2005). The modulation of brain neuropeptides, NPY, AgRP and POMC, by resistant starch is unknown, except the most recent report showing that resistant starch fed mice have high activity in hypothalamus measured by neuronal magnetic resonance imaging (MRI) (So et al. 2007).

This study investigates the role of hypothalamic neuropeptides and vagal nerves on decreasing body fat by resistant starch. The hypothesis for this work is that 1) the hypothalamic NPY/AgRP and POMC mRNA expression are altered in resistant starch fed rats; and 2) afferent vagal nerves are involved in this process. To test the hypothesis, rats' visceral afferent nerves were destroyed with a neurotoxin, capsaicin to examine whether the effect of resistant starch would be abolished. NPY, AgRP, and POMC

mRNA expressions in arcuate nucleus of hypothalamus were measured in rats fed resistant starch and control diets.

CHAPTER 2

REVIEW OF LITERATURE

Resistant Starch

The term resistant starch (RS), first coined in 1982 (Ritter et al. 1989), refers to a sum of starch that resists digestion by amylase in the small intestine and mainly reaches the large intestine where the undigested starch is fermented by the microflora to produce short chain fatty acids. RS is divided into four subcategories: RS1, RS2, RS3, and RS4. RS1 represents starch in whole grains that are in a physically inaccessible form. RS2 is a type of starch, such as ungelatinized starch, which is tightly packed in a radial pattern and resists digestion. The high amylose cornstarch used in the current study is an example of a RS2. RS3 is the type of starch that is most resistant to digestion. The starch fitting in this category is mainly retrograded amylose formed in the process of cooling the gelatinized starch and can escape the digestion of pancreatic amylase almost totally. RS4 includes structurally modified starch by chemical treatment linking amylose strands. In our study, the starch used is composed of 60% amylose and 56%RS.

Adding resistant starch to diets produces several health benefits, including lower body fat storage (Brown, 2004; Higgins, 2004). Some human studies claim that diets containing resistant starch increase satiety and decrease food intake (Achour et al. 1997; Liljeberg et al. 1999; Raben et al. 1994) and the opposite result has also been reported (de Roos et al.1995). These equivocal results are due to the lack of direct comparisons in these studies, as dietary texture and energy content used in those studies are different. In contrast to human studies, our previous works show that body fat is consistently lower in resistant starch fed animals compared to control animals fed the same dietary texture and energy density diet (Garcia et al. 2003; Hegsted et al. 2003; Keenan et al.2006). Thus,

resistant starch might be an alternative dietary carbohydrate for developing weight control diets.

The mechanism of decreased body fat by resistant starch is not completely understood. As a part of the diet, RS potentially has three major effects (Keenan et al. 2006): metabolizable energy dilution, a bulking effect and fermentation to produce short-chain fatty acids and increase PYY and GLP-1 through nutrient-gene interactions. Resistant starch dilutes the energy density of the diet, which previously was considered the main mechanism for decreased body fat by resistant starch. However, in our previous studies, we balanced the energy density in the two diets, RS and EC, to exclude the effects of energy dilution, and still obtained the similar outcome. Another assumption is that fermentation of resistant starch causes discomfort in the gut, which leads to decreased food intake and body weight. Nevertheless, resistant starch fed animals eat the same or more food than controls (Keenan et al. 2006), which indicates at most only minor effects of gut discomfort in decreased body fat. Thus, the bulking effect of fiber, which induces the decrease of food intake on account of the distension of the GI tract, might be considered not as a major factor on RS reducing body fat.

It is reported that resistant starch fed animals have significantly higher levels of peptide YY (PYY) and glucagon-like peptide -1 (GLP-1) (Keenan, et al. 2006; Zhou et al. 2006). PYY and GLP-1 are gut secreted hormones and candidates for anti-obesity drugs (Murphy et al. 2006; Young 2006). Administration of GLP-1 or PYY reduces food intake and body weight in animals and humans (Batterham et al. 2002; Drucker et al. 2006; Neary et al. 2005). These two hormones alter energy balance by sending signals from the gut to the brain, and result in brain neuropeptide expression changes (Wren et al.

2007). Therefore, the mechanism of decreased body fat by resistant starch might also relate to the similar gut-brain connection and the regulation of brain neuropeptides.

Glucagon-like Peptide-1 (GLP-1)

GLP-1 is a satiety peptide yielded from the preproglucagon gene product in the L cell of the distal intestine (Badman and Fliter. 2005)

GLP-1 has several forms in the circulation. The inactive forms, GLP-1₁₋₃₆ and GLP-1₁₋₃₇, are cleaved from preproglucagon, depending on whether the C-terminal glycine is present. Further N-terminal truncation is required to produce the biologically active forms, GLP-1₇₋₃₆ and GLP-1₇₋₃₇ (Mojsov et al. 1986).

GLP-1 is released into the circulation in a biphasic manner in proportion to the calories ingested (Orskov et al. 1994). The early phase release seems to be mediated by a neuroendocrine reflex, whereas the latter is a result of the presence of nutrients directly with L-cells.

The action of GLP-1, as a potent incretin, including mediating glucose-dependent insulinotropic effects (Holst et al. 1987), inhibiting gastric acid secretion and delaying gastric emptying, as well as promoting an increase in pancreatic β -cell mass (Tolessa et al. 1998; Naslund et al. 1999). Like other gut peptides, GLP-1 also functions within the central nervous system (CNS) as a neurotransmitter. It has been confirmed that GLP-1 receptors are distributed in a number of areas of the brain. These include the Arcuate nucleus (ARC), the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus and the area postrema of the brainstem (Wei et al. 1995; Shughrue et al. 1996), most of which are important in appetite control. A high density of GLP-1 receptors is localized in the ARC.

Both CNS-injected and peripherally administered GLP-1 inhibit food intake in rodents. Turton *et al.* ICV administered GLP-1 to rats and demonstrated a significant inhibition of food intake. The feeding effect was inhibited by the presence of exendin₉₋₃₉, a competitive antagonist to GLP-1 (Turton et al. 1996).

Similar data were obtained from human studies. GLP-1 decreases appetite and caloric intake in lean and obese humans in a dose-dependent manner (Gutzwiller et al. 1999a). Exendin-4, a potent agonist at GLP-1 receptors, also reduced food intake in healthy volunteers (Edwards et al. 2001). In a recent meta-analysis of seven studies, a significant dose-dependent decrease in appetite and caloric intake by GLP-1 infusion was shown both in lean and obese subjects (Verdich et al. 2001).

Peptide YY (PYY)

PYY is secreted by L-cells located in the gastrointestinal tract, especially in ileum, colon and rectum. There are two main forms of PYY in the circulation: PYY1–36; PYY3–36 (Batterham et al. 2002). The truncated form, PYY3-36, is created by cleavage of the N-terminal residues by dipeptidyl peptidase IV (DPP-IV) (Grandt et al. 1994). Peripheral administration of PYY has several actions, including delaying gastric emptying and gastric secretion, and increasing ileum absorption. It has also been reported that peripheral administration of PYY3–36 or injection directly into the ARC inhibit food intake and reduce weight gain in rodents (Batterham et al. 2002). However, when injected into the cerebroventricular system, PYY3-36 increases the food intake (Kanatani et al. 2000). But in Y1 and Y5 knockout mice, this effect is weakened. PYY3-36 exhibits relative specificity for the Y2 receptor. When it crosses the blood brain barrier, it probably exerts its actions via the presynaptic Y2 receptor of NPY neurons in the ARC, reducing NPY expression and consequently inhibiting feeding (Challis et al. 2003). It is

consistence with the discovery that the inhibitory Y2 autoreceptor is highly expressed on NPY neurons in the ARC (Broberger et al. 1997), whereas Y1 and Y5 receptors are localized in areas such as the PVN. Therefore, it also has been suggested that the orexigenic effects of ICV-administered PYY and PYY3-36 are mediated through Y1 and Y5 receptors. The ARC, with a relative lack of blood–brain barrier, is more exposed to circulating PYY3-36 than other areas of the hypothalamus. It has been suggested that the melanocortin system may not be essential for the mediation of the inhibitory effects of PYY3-36 on energy intake (Challis et al. 2004).

An alternative mechanism through which the anorectic effects of PYY3-36 are mediated has been proposed. Y2 receptor mRNA is also found expressed in the NTS and the nodose ganglion of the vagus nerve (Koda et al. 2005). Therefore, it suggests that PYY3-36 may inhibit feeding via the vagus. Abbott et al performed bilateral subdiaphragmatic vagotomy and transectioning of the brainstem–hypothalamic neuronal pathways. The anorectic effects of peripheral PYY3-36 was abolished in rats underwent either of these two procedures as well as *c-fos* in the ARC in response to PYY3-36 (Abbott et al. 2005).

It has been reported that PYY₃₋₃₆ can affect POMC neuron activity (Challis et al. 2003). Two studies suggested that PYY₃₋₃₆ can stimulate POMC neuron activity (Batterham et al. 2002; Challis et al. 2003). However, it still remains controversial for the effect of PYY₃₋₃₆ on POMC neuron activity: other groups have shown that PYY₃₋₃₆ inhibits rather than activates hypothalamic POMC neurons (Acuna-Goycolea et al. 2005; Ghamari-Langroudi et al. 2005). Moreover, peripheral PYY injection still induces a normal anorectic response in POMC knockout mice (Challis et al. 2004). Therefore, it still remains unclear on this issue.

Neuropeptides in Hypothalamic Arcuate Nucleus

Arcuate nucleus, locating around the third ventricle and above the median eminence, is the chief hypothalamic area involved in the control of food intake. Therefore, it is also considered as the 'master hypothalamic centre' for feeding control. Its location where the blood brain barrier is relatively deficient allows the entry of various peptides from circulation, like PYY and leptin, and modifying the activity of two populations of neuron within the ARC (Woods et al. 2003).

There are two distinct but interconnected groups of neurons in the ARC. One set of neurons distributing in the ventromedial part of the ARC release orexigenic neuropeptide Y and agouti-related peptide(Broberger et al. 1998). The other population of neurons which situate in the ventrolateral part of ARC expresses anorexigenic products of pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript (Elias et al. 1998).

These neurons expressing NPY and AgRP mostly project to the paraventricular nucleus. (Hahn et al. 1998), whereas the other set of neurons projects more broadly within the central nervous system (CNS) to hypothalamic nuclei such as the dorsomedial hypothalamic nucleus, the lateral hypothalamic area (LHA) and the PVN. Thus, AgRP/NPY and CART/POMC neurons act as the primary hypothalamic site of action of peripheral hormones. Hypothalamic nuclei, such as the PVN and the LHA, which the ARC neurons project to, act as the second order neurons.

Neuropeptide Y (NPY)

NPY is a thirty-six-amino acid peptide and is the most powerful central enhancer of appetite. It is widely distributed in the CNS with a predominant expression in ARC (Håkansson-Ovesjö et al. 2000). 90% of NPY neurons co-express AgRP (Peyron et

al.2000). NPY mRNA levels and NPY release in the ARC are enhanced in the conditions such as low leptin levels, negative energy balance, and hypoglycemia (Swart et al. 2002). Central administration of NPY alters the energy balance through reducing energy expenditure, inducing striking hyperphagia and promoting adipogenesis in rats (Pinto et al. 2004).

Although NPY is a potent orexigenic neuropeptide, NPY-knock-out mice show normal body weight and adiposity (Erickson et al. 1996). That is probably due to a compensatory and redundant mechanism in the orexigenic pathways. This is supported by the observation that adult mice underwent selective ablation of AgRP/NPY neurons became hypophagia and leanness (Gropp et al. 2005; Luquet et al. 2005).

NPY receptors are G-protein-coupled receptors. To date, six have been isolated, named Y1–Y6. (Kalra et al.1999). Most of these receptors are present in rat brain, except Y6, which is absent in rats and only found active in mice (Inui, 1999). Y1 and Y5 seem to be involved in mediating the NPY orexigenic effects (Stanley et al., 2005). Now their antagonists are under investigation as antiobesity agents.

Melanocortins

Melanocortin system is critical for the regulation of energy homeostasis: the defects in MC4 are responsible for up to 6% of monogenetic obesity in humans (MacKenzie, 2006). The melanocortin system includes neurons expressing pro-opiomelanocortin (POMC) in arcuate nucleus and nucleus of the solitary tract (NTS), NPY/AGRP neurons originating in ARC and downstream targets of these neurons expressing melanocortin receptor3 and 4 (Cone, 2005).

Among the products of cleavage of POMC is α -melanocyte-stimulating hormone, a peptide which can bind melanocortin receptor 4 (MC4) and promote energy expenditure

(Cone, 2006). Thereby Pro-opiomelanocortin (POMC) neurons are suggested to be primarily anorexigenic neurons. On the contrary to the POMC derived peptides, AgRP is an endogenous melanocortin receptor antagonist which leads to energy excess (Bagnol, 1999). Overexpression of AgRP blocks MC4 and results in obesity. AgRP is exclusively co-expressed in the NPY-containing neurons of the arcuate nucleus.

MC3R mRNA was found coexpressed in both AGRP and POMC neurons in the ARC; however, neither AGRP nor POMC cells displayed MC4R mRNA (Bagnol et al., 1999). Expression of the MC3R by POMC neurons provides a potential circuit for amplification of AGRP-mediated signals, whereas the expression of the MC3R by AGRP neurons provides a potential circuit for negative autoregulation of POMC-mediated signals. Together with other evidence such as, MC3R specific agonist inhibiting spontaneous firing of POMC neurons (Cowley et al., 2001) and increasing food intake by peripheral administration of a MC3-R selected agonist (Marks et al., 2006), MC3-R is now regarded as an inhibitory auto-receptor on the ARC melanocortin circuit.

MC4R shows intense distribution in the hypothalamic nuclei including the PVN, the DMH and the LHA (Liu et al., 2003). MC4R-overexpression in the PVN and amygdala, hyperphagia is completely reversed, whereas reduced energy expenditure is unaffected, suggesting that MC4R in the PVN specifically regulates food intake, while MC4R in other regions mainly controls energy expenditure (Balthasar et al. 2005).

Overall, the current view on hypothalamic control of energy homeostasis is that it is regulated via the balance between orexigenic NPY/AGRP neurons and anorexigenic POMC neurons.

Capsaicin

Capsaicin is the active ingredient in hot chilli peppers. It is present in large

quantities in the white pith tissue which holds the seeds. However, the seeds do not contain any capsaicin.

Capsaicin binds to a receptor called the vanilloid receptor subtype 1 (VR1), an ion channel-type receptor (winter et al., 1995). When this receptor is activated, it increases membrane permeability to cations, like calcium and sodium, allowing them to pass through the cell membrane and into the cell from outside. The capsaicin caused "depolarization" of the neuron stimulates it to signal the brain and produces a burning and painful feeling similar as that excessive heat would (Dray, 1992). But for prolonged periods of application, capsaicin would cause irreversible toxic effects resulting in the loss of sensory neurons. Although the mechanisms are unclear, it has been shown that the increase in calcium concentration may activate some calcium-dependent proteases and leads to cell death (Chard et al., 1995).

Capsaicin binds to and activates the thinly myelinated A primary sensory neurons and unmyelinated C-fibers. Intraperitoneal capsiacin destroys vagal (also some non-vagal, like trigeminal) sensory fibers (winter et al., 1995). Systemic injection with large dose of capsaicin can degenerate targeted C-fiber terminals as well as those beyond the area of interest. It also brings some adverse effects, for example, the pulmonary chemoreflex and reflex bronchoconstriction caused by the activation of the pulmonary C fibre VR1 (Nault et al., 1999) and cardiac dysfunction (Zvara et al., 2006). Perivagal application of capsaicin needs very small dose of capsaicin and isn't accompanied with systemic adverse effect (Cakir et al., 2007). However, it was observed that, with localized application of capsaicin, c-fiber degenerated distally and extensive axonal sprouting could be seen from the proximal nerve (winter et al., 1995).

CHAPTER 3

DIETARY RESISTANT STARCH INCREASES HYPOTHALAMIC POMC EXPRESSION INDEPENDENT OF CAPSAICIN-SENSITIVE NEURONS IN RATS

Introduction

Resistant starches (RS) are non-digestible, fermentable fibers that have potential to treat obesity and related disease. Our previous research showed that RS reduced body fat in rodents (Keenan et al. 2006). Also, we found RS feeding increased the gene expression and plasma concentration of peptide YY (PYY) and glucagon-like peptide -1 (GLP-1) (Zhou et al. 2006). PYY and GLP-1 are two satiety hormones that are released from the gut and signal the brain to alter the energy balance by affecting activities of two sets of neurons in the hypothalamus, NPY/AGRP and POMC. Although the essential role of NPY/AGRP and POMC in energy homeostasis is well established, it is not clear that how PYY and GLP-1 affect NPY/AGRP and POMC neurons. Some studies have shown that the effect of these two hormones could be attenuated by ablation of the vagal trunk (Abbott et al. 2005; Koda et al. 2005), while another study did not observe this effect (Osaka et al. 2005). Because RS fed animals had reduced body fat and increased expression of PYY and GLP-1 (Zhou et al. 2006), we hypothesize that 1) the hypothalamic NPY/AGRP and POMC mRNA expression may be altered by RS feeding; 2) afferent vagal nerves are involved in this process. In this study, therefore, we measured the impact of RS feeding on mRNA expression of three neuropeptides in the arcuate nucleus of the hypothalamus to investigate the mechanism of RS on reducing body fat. Additionally, we destroyed visceral afferent nerves with a neurotoxin, capsaicin, to examine whether the effect of RS would be abolished.

Methods and Materials

Animals and Diet

Fifty-two male Sprague-Dawley rats aged 7~8 weeks and weighing 150~200g at the beginning of the study, were obtained from Harlan Industries (Indianapolis, IN). They were housed individually in hanging wire-mesh cages in a temperature-controlled room (22 ± 1 °C) on a 12 h/12 h light/dark cycle with the light on at 7am. Rats were acclimated for 1 week to a powdered diet and to the cages. Water and assigned diet were available *ad libitum* during the experiment except as noted. The protocols were approved by Pennington Biomedical Research Institutional Animal Care and Use Committee.

The composition of the two experimental diets used in this study is listed in Table 1. The resistant starch (RS) diet contained 30% (weight/weight) resistant starch (Hi-Maize® cornstarch; National Starch & Chemical Co., Bridgewater, NJ). The equal energy density control (EC) diet had 100% amylopectin cornstarch (Amioca®; National Starch and Chemical Co.) as the carbohydrate source and equal energy density as RS diet (3.3kcal/g) by using non-fermentable cellulose (Dyets, Bethlehem, PA) to dilute the energy density.

Capsaicin Treatment

After one week of acclimation, rats were grouped according to weight with a randomized block design. Two groups of rats were injected intraperitoneally with either capsaicin or vehicle under inhalation anesthesia (isoflurane). The total capsaicin dose (117.5mg/kg; Sigma Chemical) was administered as a series of injections on three consecutive days in increasing doses (12.5, 30, and 75mg/kg) (Kelly et al. 2000). Capsaicin was dissolved in a mixed solution of 10% ethanol, 10% Tween 80 and 80%

Table 1. Experimental Diet Composition

Ingredients	Control		RS	
	grams	kcal	grams	kcal
100% amylopectin	424.5	1485.8	0	0
High amylose starch				
60% amylose/ 40% amylopectin	0	0	530.7	1486
Sucrose	100	400	100	400
Casein	200	716	200	716
Soybean oil	70	591.5	70	591.5
Cellulose	156.2	0	50	0
Mineral mix	35	30.8	35	30.8
Vitamin mix	10	38.7	10	38.7
Choline chloride	1.3	0	1.3	0
L-cystine	3.0	12	3.0	12
	1000 g/kg	3.3kcal/g	1000 g/kg	3.3kcal /g

sterile saline. During the injection of capsaicin, artificial ventilation and chest massage were provided to all rats who exhibited respiratory arrest, which typically occurred in the first few minutes after the injection. The survival rate during the capsaicin treatment was 70%.

The effectiveness of the capsaicin treatment was validated using the cholecystokinin (CCK) feeding-suppression test, a capsaicin-sensitive vagal nerve dependent response (Kelly et al. 2000).

CCK Feeding- Suppression Test

Four days after the last capsaicin or vehicle injection, all rats were injected intraperitoneally with either CCK or saline after an overnight fasting. Half of the capsaicin and vehicle treated rats received CCK (6ug/kg), and the other half received the same volume of saline five minutes prior to given access to food. Then food intake was measured for the following 30 minutes. Three days later, the same test was repeated except that the rats receiving CCK previously were injected with saline, and the rats that received saline previously were injected with CCK. In the vehicle-treated rats, the administration of CCK significantly suppressed 30 minute food intakes in overnight fasted rats (5.22 ± 0.14 vs. 2.86 ± 0.17 ; $p < 0.001$). But all capsaicin treated rats failed to respond to CCK and did not reduce food intake (4.63 ± 0.19 vs. 4.18 ± 0.15 ; $p = 0.12$).

Experimental Design

Nine days after the capsaicin treatment, both capsaicin and vehicle-treated rats were divided into two diet treatment groups, resistant starch and energy control, by randomized block design based on their weight. The four groups of rats were fed their assigned diets for 65 days. Food intake and body weight were measured three times per

week throughout the experiment. After 65 days, the animals were sacrificed via decapitation. Different fat pads (epididymal fat, perirenal fat, and remaining fat in the abdominal area, defined as abdominal fat) were removed and weighed. Total body fat used for body fat calculation was the sum of epididymal fat, perirenal fat, and abdominal fat. The gastrointestinal (GI) tract was removed and weighed after removal of mesenteric fat. Disemboweled weight was calculated by subtracting GI weight from body weight.

Plasma Assays

Blood was collected in EDTA tubes with final EDTA concentration 1.8mg/ml blood and centrifuged at 4000 X g for 20 minutes to extract plasma. Plasma PYY and GLP-1 were measured by radioimmunoassay with RIA kits from Linco Research Inc. (St. Louis, MO).

To make the standard curve for PYY, 100 ul of the six standards (15.6-500pg/ml) was mixed with 100ul PYY antibody, and 300ul assay buffer in tubes to incubate overnight at 4 °C. On the second day, 100 ul ¹²⁵I-Rat PYY was added into the mixture and incubated overnight at 4 °C. On day three, 10 ul Rabbit Carrier and 1.0 cold precipitating Reagent were pipetted into the tubes in turn. After following incubation and centrifugation, supernatant was decanted from the tubes and radiation counts were determined with gamma counter. The counts were regressed on the PYY standard concentration to obtain the standard curve. For the sample measurement, the same procedure was performed. The PYY concentration in each sample was calculated using the standard curve, expressed in pg/m.

To make the standard curve for GLP-1, 100 ul of the seven standards (10-1000pM) was mixed with 100ul GLP-1 antibody and 400ul assay buffer in tubes to incubate

overnight at 4 °C. On the second day, 100 µl 125I-Rat GLP-1 was added into the mixture and incubated overnight at 4 °C. On day three, 10 µl Rabbit Carrier and 1.0 cold precipitating Reagent were pipetted into the tubes in turn. After following incubation and centrifugation, supernatant was decanted from the tubes and radiation counts were determined with gamma counter. The counts were regressed on the GLP-1 standard concentration to obtain the standard curve. For the sample measurement, the same procedure was performed. The GLP-1 concentration in each sample was calculated using the standard curve, expressed in pM.

Microdissection of Arcuate Nucleus (ARC) in Hypothalamus

Brains from decapitated rats were quickly removed, frozen on dry ice and stored at -70 °C. The middle brain was dissected using a cryostat. Microdissection of the ARC was performed using the procedure described by Palkovits (Palkovits, 1988). Five continuous coronal sections were collected starting from Bregman -2.12mm to -3.4mm for the ARC micropunch. The thicknesses of sections were 300µm each. The micropunch was performed bilaterally under a microscope, using a needle (Stoelting, Chicago, IL) with an inner diameter of 0.51mm. Immediately after each punch, the tissue was put into 100 µl of ice-cold 2- mercaptoethanol-lysis buffer (ratio is 0.7:100), and vortex until the sample was homogenized. Then equal volumes of cold 70% ethanol were added to the lysate and mixed thoroughly for 10seconds. The mixture was then stored at -70 °C until we began to perform the RNA extraction.

Measurements of NPY, AGRP and POMC mRNA Expression

RNA was extracted from micro-punched tissue using Absolutely RNA microprep kit from Stratagene (La Jolla, CA). The ethanol-lysate mixtures from one rat was

vortexed again for 5 sec, transferred to a RNA-binding cup that was sat on a 2-ml collection tube, and centrifuged for 1 min at 18,000 x g at 4 °C. All the following spin condition is 18,000 x g at 4 °C. The filtrate was discarded and 600 ul of low-salt wash buffer was added to the cup, followed by 1 min centrifuge. After discarding the filtrate, and spinning the cup for another 2 min to dry the fiber matrix, 30 ul of RNase-free DNase I solution was pipetted directly onto the fiber matrix. Then the cup was incubated in a 37 °C water bath for 15 min. The RNA was washed with 500 ul of high-salt, 600 ul and 300 ul of low-salt wash buffer respectively; one-minute spin was applied after each wash. Following the final wash the cup was spun for 2 min to dry the fiber matrix. Then the cup was moved to a 1.5-ml collection tube, and 20 ul of elution buffer was added directly onto the fiber matrix. After a 2-min incubation at room temperature, the cup was spun for 1 min. For RNA quantification, 1.5 ul of sample RNA was used to detect the optical density (OD) 260 and OD280 using a nanodrop.

The gene transcription for AgRP, NPY, and POMC in the ARC of the hypothalamus was determined using real-time reverse transcriptase polymerase chain reaction, and results were expressed as a ratio to the expression of the constitutive gene cyclophilin. The sequences of the primers and probes for rat cyclophilin, NPY and AGRP were listed in Table 2. The probe and primers for POMC (assay identification no. Rn00595020_ml) were purchased from Applied Biosystems(Foster City, CA).Real time RT-PCR reaction mixture was 10 ul of total volume, including 9ng of sample RNA, 1 ul of 10 X Tagman buffer, 5.5mM MgCl₂, dATP, dCTP, dUTP and dGTP each 0.3 mM, 500 nM forward primers, 500 nM reverse primers, 200 nMTaqman probes, 7.5 U RNase inhibitor, 5 U MuLV reverse transcriptase, 0.3 U AmpliTaq Gold DNA polymerase and

Table 2. The sequences of primers and probes for real time RT-PCR. F: forward primer, R: reverse primer, P: Taqman probe, CYC: cyclophilin, NPY, neuropeptide Y, AgRP: agouti-related peptide.

Gene	Sequence	Genebank
Rat CYC	F: 5'CCCACCGTGTTCTTCGACAT3' R: 5'TGCAAACAGCTCGAAGCAGA 3' P: 5'CAAGGGCTCGCCATCAGCCG 3'	M15933
Rat NPY	F: 5' TCTGCCTGTCCCACCAATG 3' R: 5' CAACGACAACAAGGGAAATGG3' P: 5' CCACCACCAGGCTGGATTCCGA 3'	M20373
Rat AGRP	F: 5' TTGGCAGAGGTGCTAGATCCA 3' R: 5' AGGACTCGTGCAGCCTTACAC 3' P: 5' CGAGTCTCGTTCTCCGCGTCGC 3'	AF206017

RNase-free H₂O. Each sample was tested in duplicate. The one-step real-time reverse transcriptase polymerase chain reaction condition is 48 °C for 30 min, 95°C for 10 min for one cycle, 95 °C for 15 sec and 60 °C for 1 min for 40 cycles.

Statistical Analysis

Data are presented as means \pm SEM. Statistical analyses were performed using the Statistical Analysis System (SAS 9.1). A factorial arrangement of the treatments (two-way ANOVA) was used to examine the influence of the two main effects of diet and capsaicin/vehicle treatment on all measurements. Subgroup means were compared by Tukey's method.

Results

POMC, NPY, and AgRP mRNA expression in arcuate nucleus of hypothalamus

POMC expression in the ARC (Figure 1) was significantly up-regulated by dietary resistant starch ($p < 0.05$). Capsaicin treatment did not affect the influence of resistant starch on POMC expression ($p > 0.05$). There were no effects of dietary resistant starch or capsaicin injection on expression of NPY and AgRP (Figure 1).

Fat Pads Weights

Compared with rats fed the control diet, dietary resistant starch significantly decreased total body fat and fat/disemboweled weight in both the vehicle and capsaicin groups. There was no interaction between diet and treatment ($p > 0.05$), although both diet and treatment had an effect on these two measures (diet $p < 0.001$, treatment $p < 0.01$) (Figures 2).

Plasma PYY and GLP-1 Concentrations

Capsaicin treatment did not affect the increase of plasma PYY and GLP-1

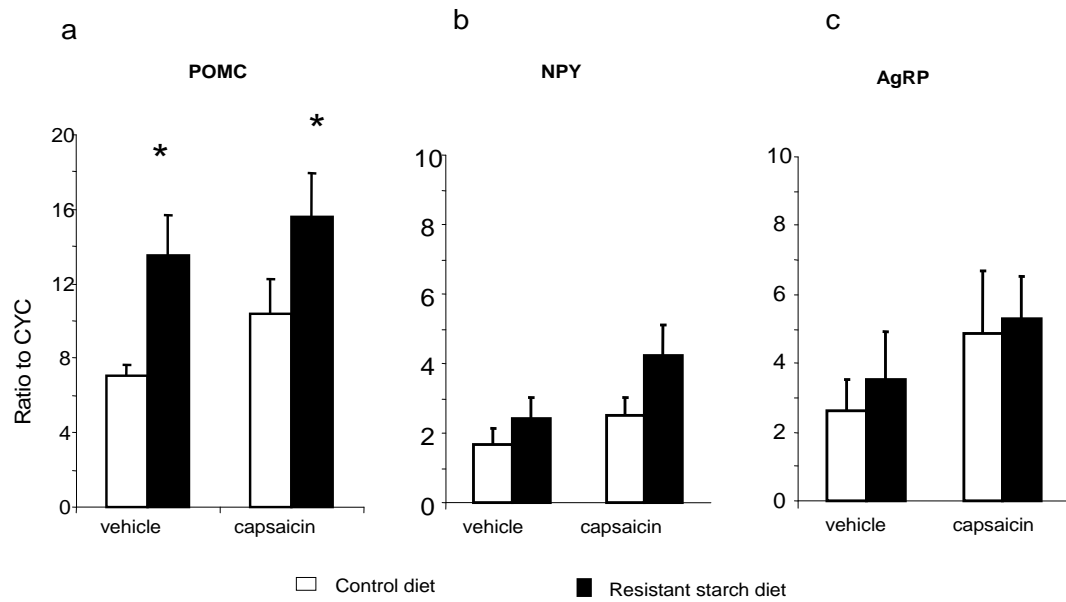


Figure 1. Dietary resistant starch increases POMC (a), but not NPY (b) and AgRP (c) mRNA expressions in arcuate nucleus of resistant starch fed rats treated with vehicle or capsaicin. Data are mean \pm SEM for group of 7-9 rats. For POMC mRNA expression, diet: $P < 0.05$, capsaicin: $P > 0.05$, Interaction: $P > 0.05$ by two way ANOVA. For NPY and AgRP, there were no significant effects on diet, capsaicin, and interaction. * $P < 0.05$ vs. controls within the same treatment (vehicle or capsaicin).

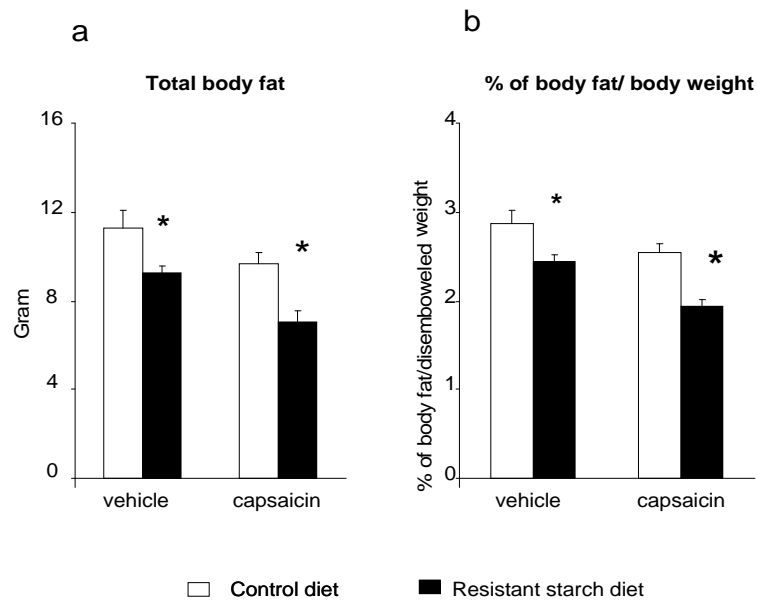


Figure 2. Total body fat (a) and percentage of body fat/ disemboweled body weight (b) were decreased in resistant starch fed rats treated with vehicle or capsaicin. Data are mean \pm SEM for group of 10-11 rats. Two way ANOVA analysis indicates there were significant diet ($p < 0.001$) and a significant capsaicin treatment effects ($p < 0.01$), with no interaction effect. * $P < 0.05$ vs. controls within the same treatment (vehicle or capsaicin)

concentrations (treatment $p>0.05$, diet $p<0.001$) induced by RS feeding. (Figures 3)

Food intake and disemboweled weight

There were no statistical differences of food intake between control and RS fed rats. It demonstrated no or minimal discomfort with the consumption of resistant starch at the levels in their diet. Because RS fed rats had significantly heavier GI contents, the disemboweled body weight was used to exclude GI contents from body weight. There was no significant difference for disemboweled body weight between control and RS fed rats within capsaicin or vehicle treatment groups. (Table 3)

Discussion

In this study, we investigate the mechanism of decreased body fat by dietary resistant starch. We demonstrate that dietary resistant starch increases hypothalamic POMC expression independent of capsaicin-sensitive neurons in rats. Specifically, we measured mRNA expressions of POMC, NPY and AgRP in the arcuate nucleus in the context of resistant starch feeding and capsaicin treatment. To our knowledge, our finding provides the first direct evidence that dietary resistant starch alters brain neuropeptide expression in rats.

Feeding resistant starch significantly up-regulated the expression of POMC, but had no effect on the expressions of NPY and AgRP in rats. These results are consistent with the observation that food intake is similar between control and RS fed rats in our study. Actually, RS fed rats have a tendency to eat more food. Although studies showing that resistant starch fed animals decreased energy intake compared to the control diet fed animals, we noticed that the control diet used in those studies had higher energy density than the RS diet (So et al. 2007). In human subjects, there are uncertain and

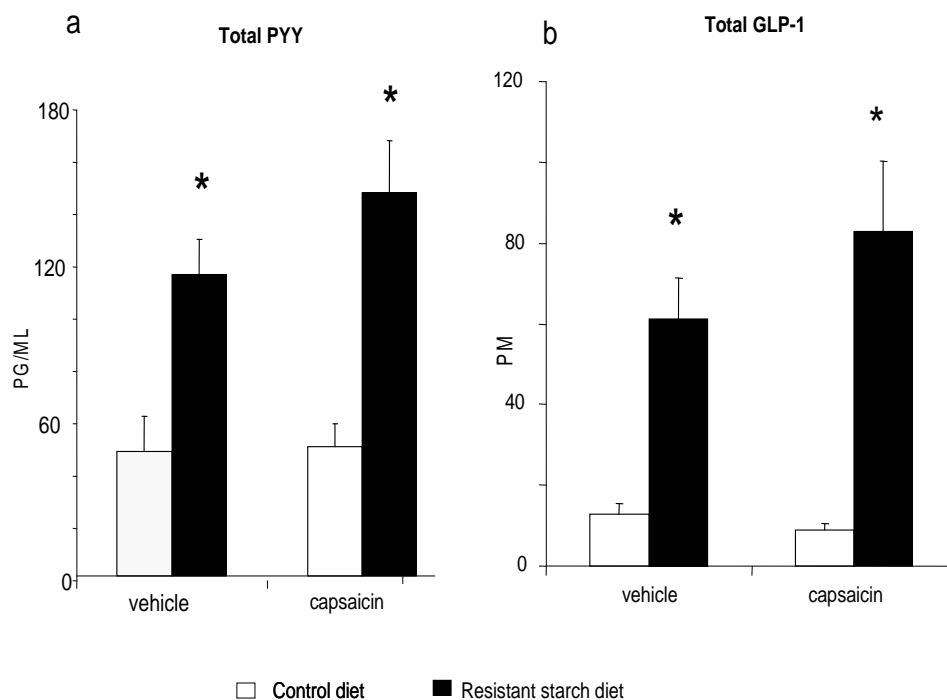


Figure 3. Plasma total PYY (a) and total GLP-1 (b) concentrations were increased in rats fed resistant starch. Data are mean \pm SEM for group of 10-11 rats. For both 3(a) and 3(b), there was a significant diet effect ($p < 0.001$) but not a capsaicin treatment effect ($p > 0.05$) and no interaction effect ($p > 0.05$). * $P < 0.05$ vs. controls within the same treatment (vehicle or capsaicin).

Table 3 Food intakes and body weight in resistant starch fed rats treated with capsaicin or vehicle.

Group	Cumulative food intake (g)	Disemboweled body weight (g)
Vehicle-C	1256.6 \pm 9.0	391.3 \pm 7.22
Vehicle -RS	1302.6 \pm 9.8	379.1 \pm 9.6
Capsaicin-C	1380.5 \pm 15.6	377.75 \pm 7.53
Capsaicin-RS	1403.9 \pm 9.8	360.3 \pm 11.7

Vehicle-C: vehicle treated rats fed control diet

Vehicle-RS: vehicle treated rats fed resistant starch diet

Capsaicin-C: capsaicin treated rats fed control diet

Capsaicin-RS: capsaicin treated rats fed resistant starch diet

There were no significant difference in food intake and disemboweled body weight between control and RS fed rats. However, rats treated with capsaicin had lower disemboweled body weights compare to vehicle-treated rats. Data are mean \pm SEM for group of 10-11 rats

contradictive reports on the satiety effects of RS (Higgins et al. 2004), but fatty acid oxidation is significantly increased after consumption of resistant starch (Higgins et al. 2004).

Thus, the decreased body fat in RS fed rats is most likely the result of increased energy expenditure and activation of POMC neurons, rather than from decreased food intake via altering NPY/AgRP neurons.

The increased POMC and decreased body fat in RS fed rats are independent of capsaicin sensitive vagal nerves because the capsaicin treatment did not block any resistant starch effects tested in the present study. In vehicle treated rats, dietary resistant starch decreased body fat and increased plasma PYY and GLP-1 levels, which is consistent with our previous publications (Keenan et al. 2006; Zhou et al. 2006). Interestingly, the effect of RS was retained in rats when their vagal nerves were destroyed by capsaicin, implying the signals generated from the gut act directly on the brain, not via the vagal nerve. Still, there is a small chance that non-capsaicin sensitive vagal nerves can convey signals from the gut to the brain, because capsaicin only destroys small, unmyelinated primary sensory vagal afferent nerves (Ritter et al. 1989). Regardless, our results indicate that the effects of resistant starch on body fat and hypothalamic POMC gene expression do not rely on the involvement of capsaicin sensitive nerves.

Our results bring an interesting question: what causes the increased POMC expression in resistant starch fed rats? Resistant starch potentially has three major effects as a part of the diet: metabolizable energy dilution, a bulking effect, and fermentation to produce short-chain fatty acids and increase PYY and GLP-1 (Keenan et al. 2006). In our study, control and resistant starch diets have the same energy density, so the energy

dilution effect can be excluded. The bulking effect is due to the high fiber content in resistant starch diet. If the bulking causes the changes in POMC, destroying vagal afferent nerves should prevent the changes, as distension signals from the gut to the brain are vagal afferent nerve dependent (Phillips et al. 2000). But our results indicate otherwise. Thus, the mechanism of increased POMC was narrowed down to the fermentation of resistant starch and the subsequent increases of PYY and GLP-1. Further studies are needed for a conclusive determination for the cause of increased POMC in resistant starch fed animals.

Another question raised from our results is why RS fed rats do not decrease food intake despite having higher PYY and GLP-1? Our previous unpublished results indicate a broader gut-secreted hormone profile is changed by dietary resistant starch. We suspect that the other hormones/factors modulated by resistant starch may oppose the effects of PYY and GLP-1 on food intake. Additionally, both PYY and GLP-1 have active and inactive forms (Wren et al. 2007; Baggio et al. 2007). Total PYY and GLP-1 were increased in our study, while PYY and GLP-1 reduction of food intake is based on injecting active forms of PYY and GLP-1 (Neary et al. 2005; Batterham et al. 2003).

PYY has two forms: PYY₁₋₃₆ and PYY₃₋₃₆. When PYY₁₋₃₆ is released from L-cells of the ileum and large intestine, it is quickly converted to PYY₃₋₃₆ by the enzyme dipeptidyl peptidase-IV (DPP-IV) (Wren et al. 2007). PYY₁₋₃₆ and PYY₃₋₃₆ have counteracting effects on food intake. PYY₃₋₃₆ has been shown to inhibit appetite and decrease food intake by binding to Y2-receptors and exerting a negative impact on the NPY neuron (Batterham et al. 2002). In contrast, central injection of PYY₁₋₃₆ prompts food intake through an Y1-receptor mediated action (Ballantyne, 2006). Our

unpublished data have showed that the consistent higher plasma total PYY was observed over 24 hours in RS fed rats, suggesting a continuously-released pattern for PYY in RS fed rats. This release pattern is different from meal-stimulated PYY releases. Thus, the ratio of PYY₁₋₃₆ and PYY₃₋₃₆ may be high in RS fed animals and the counteracting effects of these two peptides would account for the lack of food intake differences between RS fed rats and controls.

PYY₃₋₃₆ and GLP-1 can also directly affect POMC neuron activity (Challis et al. 2003; Ma et al. 2007). Two studies suggested that PYY₃₋₃₆ can stimulate POMC neuron activity (Batterham et al. 2002; Challis et al. 2003). However, it still remains controversial for the effect of PYY₃₋₃₆ on POMC neuron activity: other groups have shown that PYY₃₋₃₆ inhibits rather than activates hypothalamic POMC neurons (Acuna-Goycolea et al. 2005; Ghamari-Langroudi et al. 2005). Moreover, peripheral PYY injection still induces a normal anorectic response in POMC knockout mice (Challis et al. 2004). Therefore, effects of resistant starch on stimulating POMC expression are more likely explained by elevated GLP-1 in RS fed animals. This deduction is based on a combination of our results and the following evidence. First, GLP-1 receptors are found located in the ARC where they overlap hypothalamic POMC neurons' residency (Merchenthaler et al. 1999). Second, GLP-1 excites POMC neurons postsynaptically via interaction with GLP-1 receptors in POMC cells from mouse ARC brain slices (Ma et al. 2007). Third, our RS fed rats had decreased body fat without reduced energy intake compared to controls. This indicates that there was increased energy expenditure in RS fed rats, and GLP-1 increased energy expenditure (Osaka et al. 2005). Further studies are

needed to block the GLP-1 action to determine if changes on POMC in RS fed rats could also be blocked.

In conclusion, the mechanism of decreased body fat by resistant starch is linked to increased neuropeptide POMC gene expression in the hypothalamus and such an effect is independent of involvement of visceral afferent capsaicin-sensitive neurons. Our findings provide a further understanding of how resistant starch works as a dietary ingredient to reduce body fat.

CHAPTER 4

CONCLUSIONS

The work in this thesis focuses on the role of hypothalamic neuropeptides and vagal nerves on decreasing body fat by resistant starch. We measured mRNA expressions of POMC, NPY and AgRP in the arcuate nucleus in the context of resistant starch feeding and capsaicin treatment. We showed that Feeding resistant starch significantly up-regulated the expression of POMC, but had no effect on the expressions of NPY and AgRP in rats. We further demonstrated that the capsaicin treated rats had a similar decreased body fat and elevated plasma PYY and GLP-1 levels as vehicle treated rats.

We provide evidence to indicate that the mechanism of decreased body fat by resistant starch is linked to increased neuropeptide POMC gene expression in the hypothalamus and such an effect is independent of involvement of visceral afferent capsaicin-sensitive neurons. Further work is needed to verify whether dietary resistant starch reduce body fat by increasing energy expenditure; the role of PYY and GLP-1 in resistant starch's effect; and the application to human.

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VITA

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